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The 3′-Terminal Consensus Sequence of Rotavirus mRNA Is the Minimal Promoter of Negative-Strand RNA Synthesis

MELISSA J. WENTZ,[1] JOHN T. PATTON,[2] AND ROBERT F. RAMIG[1]*

Division of Molecular Virology, Baylor College of Medicine, Houston, Texas 77030,[1] and Laboratory of Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892[2]

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We used an in vitro template-dependent replicase assay (D. Chen, C. Zeng, M. Wentz, M. Gorziglia, M. Estes, and R. Ramig. J. Virol. 68:7030–7039, 1994) to identify the *cis*-acting signals required for replication of a genome segment 9 template from the group A rotavirus strain OSU. The replicase phenotypes for a panel of templates with internal deletions or 3′-terminal truncations indicated that no essential replication signals were present within the open reading frame and that key elements were present in the 5′ and 3′ noncoding regions. Chimeric constructs containing portions of viral sequence ligated to a nonviral backbone were generated to further map the regions required for in vitro replication of segment 9. The data from these constructs showed that the 3′-terminal seven nucleotides of the segment 9 mRNA provided the minimum requirement for replication (minimal promoter). Analysis of additional chimeric templates demonstrated that sequences capable of enhancing replication from the minimal promoter were located immediately upstream of the minimal promoter and at the extreme 5′ terminus of the template. Mutational analysis of the minimal promoter revealed that the 3′-terminal -CC residues are required for efficient replication. Comparison of the replication levels for templates with guanosines and uridines at nucleotides −4 to −6 from the 3′ terminus compared with levels for templates containing neither of these residues at these positions indicated that either or both residues must be present in this region for efficient replication in vitro.

The rotavirus genome consists of 11 segments of double-stranded RNA (dsRNA) ranging from 667 (segment 11) to 3,302 (segment 1) bp in length. The genome along with VP1 and VP3 is present within the innermost capsid shell, which is composed of VP2, and these components collectively constitute the viral core. The core is surrounded by an intermediate shell consisting of 260 trimers of VP6 (27). These particles, referred as double-layered particles, possess transcriptase activity in vitro and in vivo (2, 28). The infectious virus particles, known as triple-layered particles, contain the double-layered particle covered with 780 monomers of VP7 and 60 VP4 dimers (30).

The rotavirus genome segments consist of complementary strands of RNA that are base paired from end to end (15). The plus strand of the dsRNA contains a 5′-terminal m^7GpppG^m cap but lacks a 3′-terminal poly(A) tail (15, 20). Each genome segment (except segment 11) contains a single open reading frame (ORF) flanked by relatively short 5′ and 3′ noncoding regions (NCRs). Although the NCRs differ in sequence and in length among the different RNA segments, they are well conserved for a particular genome segment. The roles of the NCRs have not been clearly defined; however, they are believed to provide signals (sequence specific or structural) for packaging and/or replication and may also play a role in gene expression. Consensus sequences, common to all 11 genome segments, are present at the 5′ and 3′ termini. In group A rotaviruses, the 5′-terminal consensus sequence is 5′-GGC(A/U)(A/U)U(A/U)A(A/U)(A/U)-3′ and the 3′-terminal consensus is 5′-U(G/U)(U/G)(G/U)(A/G)CC-3′.

Subviral complexes, termed replication intermediates (RIs), have been isolated from infected cells. These complexes were classified into three types, precore RI, core RI, and double-shelled RI, on the basis of distinct differences in size and protein profiles. All of the RIs analyzed had a full complement of viral mRNA and various combinations of structural and nonstructural proteins. When tested in an in vitro polymerase assay, the core and double-shelled RI types were able to continue synthesis of a nascent strand of RNA present in the complex (11). These studies showed that the synthesis of the rotavirus genome was an asymmetric process in which the viral mRNA served as template for the synthesis of minus-strand RNA (22) and that the dsRNA product remained associated with a protein complex that eventually matured into a more organized structure (11).

An in vitro template-dependent replicase system for rotavirus has recently been described (5). Replicase activity was associated with particles consisting of VP1, VP2, and VP3. These particles could be obtained from a stepwise disassembly of native virions or by the purification of virus-like particles from *Spodoptera frugiperda* (Sf9) cells infected with recombinant baculoviruses. Templates could be either native mRNAs purified from double-shelled particles with associated in vitro transcriptase activity (2, 6) or transcripts with bona fide 5′-GG. . . and . . .CC-3′ termini produced in runoff transcription reactions using rotavirus cDNA clones as templates. Analysis of the RNA product by primer extension and strand separation gel electrophoresis demonstrated that a full-length minus-strand RNA is synthesized by using the mRNA as template (31). Unlike the replicase activity obtained from the subviral complexes recovered from infected cells, this system supported both the initiation and the synthesis of minus-strand RNA.

Here we report the application of this system to identify the *cis*-acting elements required for in vitro replication of rotavirus strain OSU genome segment 9.

* Corresponding author. Mailing address: Division of Molecular Virology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-4830. Fax: (713) 798-3586. Electronic mail address: rramig@bcm.tmc.edu.

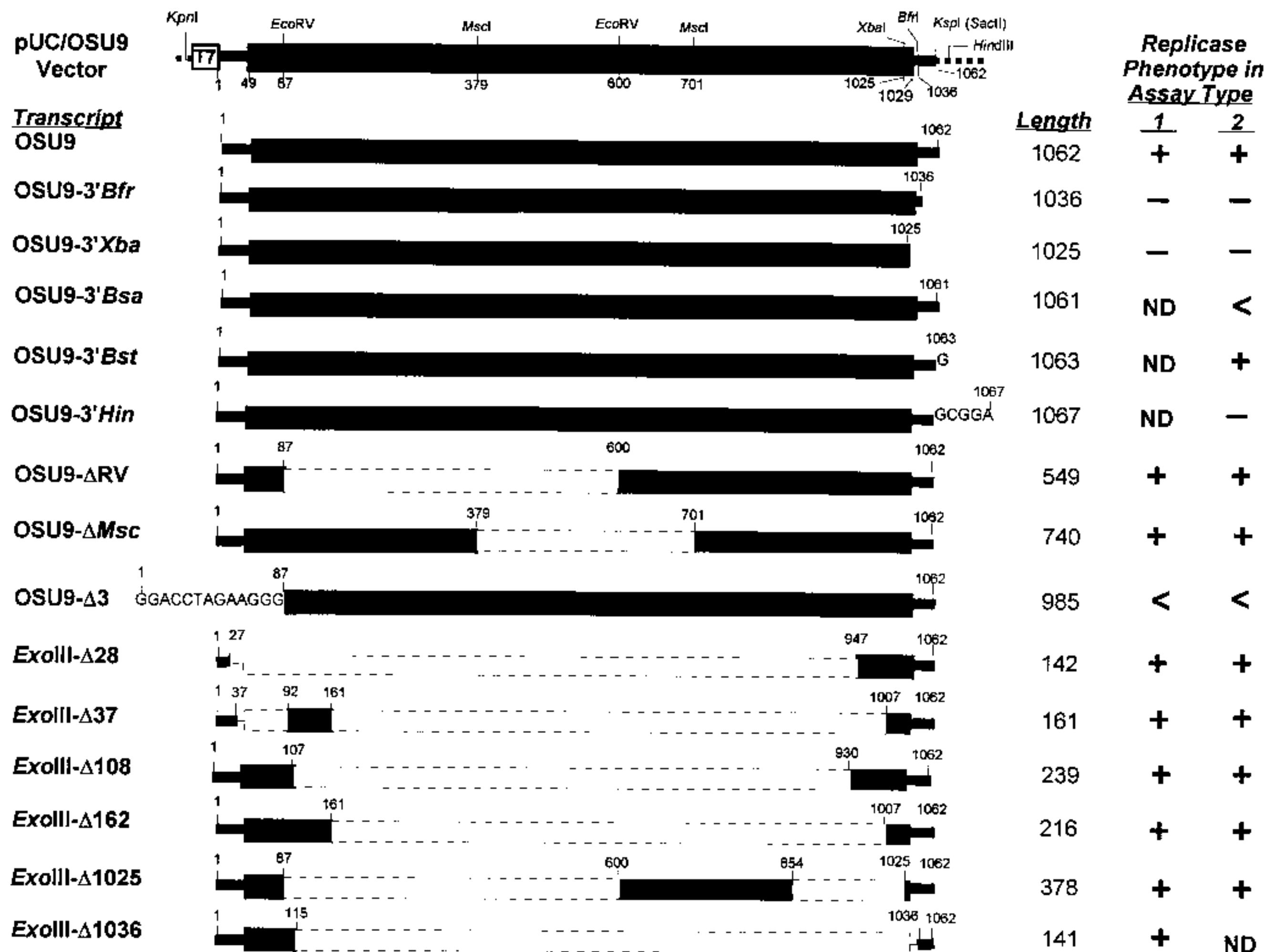

FIG. 1. Schematic diagram of the pUC/OSU9 vector and transcripts containing internal deletions or 3′-terminal truncations and their replicase phenotypes for assays types 1 and 2. Shown at the top is the cDNA clone (pUC/OSU9) for genome segment 9 of porcine rotavirus OSU strain. The locations of the T7 promoter and the relevant restriction endonuclease sites used to create internal deletions or prepare DNA for in vitro transcriptions assays are indicated. Shown below are the structures of transcripts made from the cDNA clones derived from the parental pUC/OSU9 vector as described in Materials and Methods. These transcripts contain either internal deletions, 3′-terminal truncations, or altered 3′ termini. Wide bars represent the ORF, and narrow bars represent the 5′ and 3′ NCRs. Open boxes and dashed lines represent regions absent from cDNA clones containing internal deletions. Numbers decorating each transcript indicate the nucleotide positions contained within each construct. At the right is shown the length of each transcript in nucleotides. At the extreme right are shown the replication phenotypes of the constructs as determined in assay type 1 or assay type 2. The scoring procedure used to define the replication phenotypes is described in the text.

MATERIALS AND METHODS

Cell culture and virus propagation. MA104 rhesus monkey kidney cells were grown in medium 199 containing 5% fetal bovine serum and used to propagate simian rotavirus SA11-4F at low multiplicity (0.1 PFU per cell).

Preparation of open SA11-4F core particles. Native core particles were prepared as described previously (3, 4) from purified SA11-4F virions. Open particles were produced by dialysis of native core particles against low-ionic-strength buffer (2 mM Tris-HCl [pH 7.2], 0.5 mM EDTA, 0.5 mM dithiothreitol) for 12 to 20 h at 4°C. Protein concentration was determined by using a Bio-Rad protein assay kit.

Generation of cDNA clones containing internal deletions. A PCR-generated cDNA clone (pUC/OSU9) of genome segment 9 from porcine rotavirus strain OSU was cloned into pUC19 such that in vitro transcription of *Ksp*I-cut plasmid would yield full-length mRNA with native 5′ and 3′ termini (5) (Fig. 1, top). The internal deletion construct pOSU9-ΔRV was generated as described previously (5). An additional cDNA clone was prepared by restriction endonuclease digestion followed by religation, whereas several cDNA clones were generated by exonuclease III digestion (see below). Restriction endonucleases, T4 DNA polymerase, T4 DNA ligase, exonuclease III, and mung bean nuclease were purchased from New England Biolabs and used according to the manufacturer's protocols. The final digestion products from either method were excised from a low-melting-point agarose gel and religated by using T4 DNA ligase. Ligation reactions were used to transform *Escherichia coli* DH5α as instructed by the manufacturer (Life Technologies, Inc.). The nucleotide sequences of the resulting clones (Fig. 1) were confirmed by dideoxynucleotide sequencing using a Sequenase version 2.0 kit (United States Biochemical) and appropriate oligonucleotides as primers (29).

The pOSU9-Δ*Msc*I internal deletion clone was prepared by digesting the pOSU9 clone with the restriction endonuclease *Msc*I. The pOSU9-Δ3 clone was made by partially digesting the pUC/OSU9 clone with *Eco*RV followed by *Hin*dIII digestion. The 975-bp fragment released from the double digest was gel purified and ligated into the vector v20 (a pGem4/pMJ5 hybrid plasmid [1]) which had been digested with *Sma*I and *Hin*dIII. The resulting clone contains the sequence GGACCTAGAAGGG between the T7 promoter and the OSU9 sequence.

To generate clones exoIII-Δ28, exoIII-Δ108, exoIII-Δ162, and exoIII-Δ1036, 5 μg of supercoiled pOSU9-Δ*Msc* was redigested with *Msc*I. To generate clone exoIII-Δ1025, 5 μg of supercoiled pOSU9-ΔRV was digested with *Xba*I; for clone exoIII-Δ37, 5 μg of supercoiled exoIII-Δ162 was digested with *Eco*RV. After complete digestion, the reactions were extracted with an equal volume of phenol-chloroform and subjected to ethanol precipitation. The recovered products were incubated in the presence of 100 U of exonuclease III at 30°C for 2 min. During the incubation time, aliquots were removed every 15 s and set on dry ice. Following heat inactivation of the exonuclease, all aliquots were subsequently treated with 3 U of mung bean nuclease at 30°C for 30 min. The termini generated from the digestions were polished by using T4 DNA polymerase.

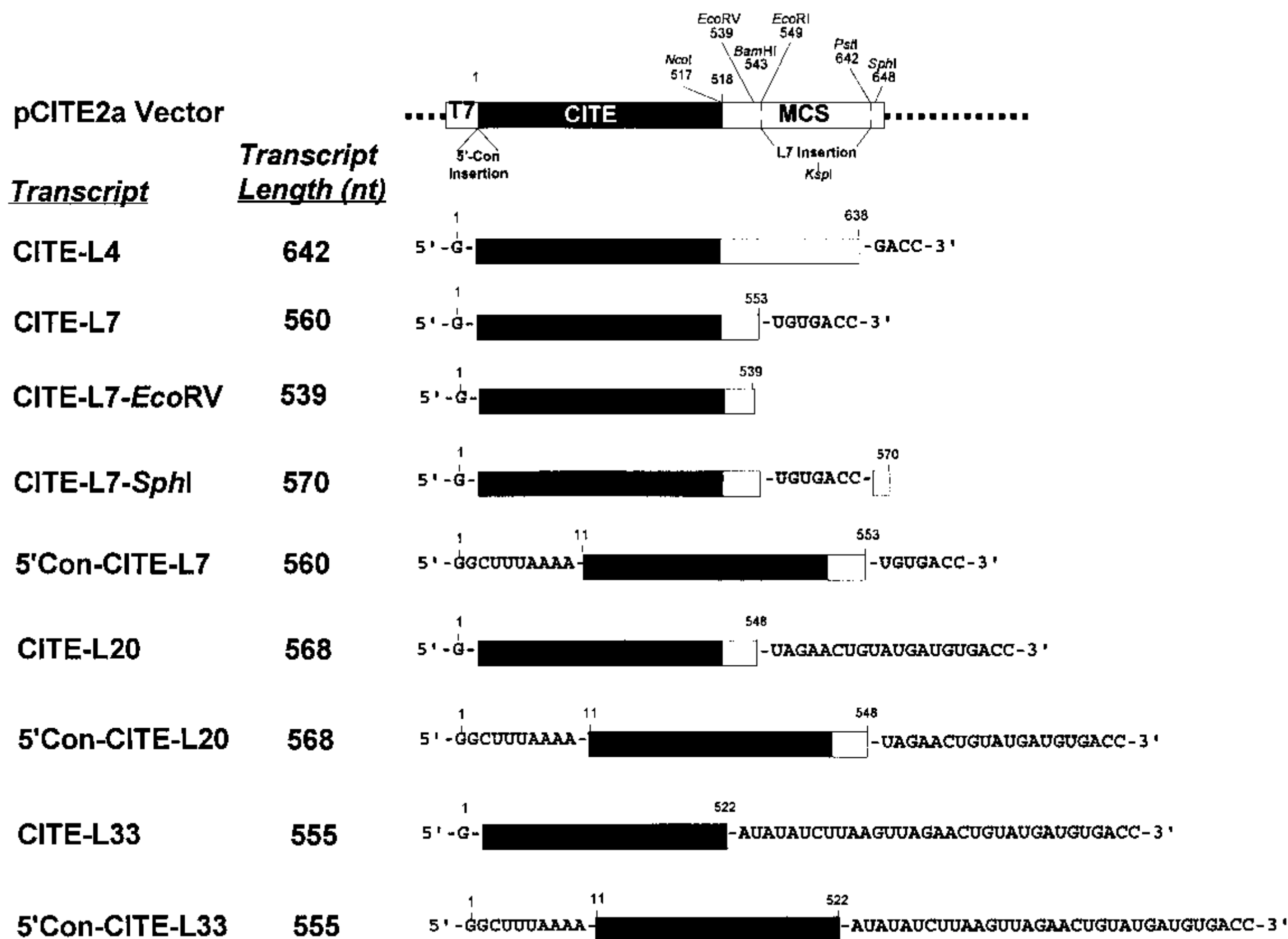

FIG. 2. Schematic diagram indicating the structures of chimeric templates. Shown at the top is the relevant region of the pCite2a cloning vector (Novagen) used to generate the chimeric cDNA clones. The CITE sequence is the cDNA of the 5′ NCR of the encephalomyocarditis virus, which contains an internal ribosome entry site. Indicated are the T7 promoter, the position of the 517-nt CITE sequence, the position of the multiple cloning site (MCS), and the positions of the relevant restriction endonuclease sites used to insert viral cDNA sequence. Shown below are the structures of the transcripts from the chimeric cDNA clones or DNA templates generated via PCR as described in Materials and Methods. The viral sequences present in each template are shown as sequence rather than boxes. The length of each transcript in nucleotides is indicated.

Digestion products were excised from a low-melting-point agarose gel and used to generate cDNA clones as described above.

Cloning strategy for chimeric constructs. The pCite-L4 construct (Fig. 2) was generated by digesting the vector pCite2a (Novagen) with *Pst*I. The 3′ overhang was removed by using T4 polymerase so that runoff transcripts would terminate with GACC-3′ (Fig. 2).

Three pairs of plus- and minus-sense oligonucleotides were synthesized and used to make three chimeric cDNA clones. Each oligonucleotide pair consisted of complementary viral cDNA sequence flanked by nonviral sequence. An adapter was made from each pair by mixing equimolar amounts of the plus- and minus-sense oligonucleotides, incubating the mixture at 100°C for 15 min, then allowing the mixture to cool to room temperature. T4 DNA ligase was used to ligate the annealed oligonucleotides to the pCite2a vector that had been digested with the appropriate restriction endonucleases and subsequently gel purified. The ligation products were then used to transform *E. coli* DH5α. The presence of the viral cDNA sequences in the cDNA clones was confirmed by dideoxynucleotide sequencing.

pCite-L7. The pCite-L7 cDNA clone contains nucleotides (nt) 1056 to 1062 of pOSU9. The plus- and minus-sense oligonucleotides used to make this construct were 5′-aattTGTGACCgcggtgca-3′ and 5′-ccgcGGTCAC-3′, respectively (viral cDNA sequence is in capital letters, a *Ksp*I site is underlined, and nonviral sequence is in lowercase letters). The annealed oligonucleotides, which formed 5′-terminal *Eco*RI and 3′-terminal *Pst*I cohesive overlaps, were ligated to the pCite2a vector which had been digested with both *Eco*RI and *Pst*I (Fig. 2).

pCite-L20. The pCite-L20 cDNA clone contains nt 1043 to 1062 of pOSU9. The plus- and minus-sense oligonucleotides used to make this construct were 5′-gatccTAGAACTGTATGATGTGACCgc-3′ and 5′-GGTCACATCATACA GTTCTAg-3′, respectively (viral cDNA sequence is in capital letters, a partial *Ksp*I site is underlined, and nonviral sequence is in lowercase letters). The annealed oligonucleotides, which formed 5′-terminal *Bam*HI and 3′-terminal *Ksp*I cohesive overlaps, were ligated to the pCite-L7 cDNA clone which had been digested with both *Bam*HI and *Ksp*I (Fig. 2).

pCite-L33. The pCite-L33 cDNA clone contained nt 1030 to 1062 of pOSU9. The plus- and minus-sense oligonucleotides used to make this construct were 5′-catggATATATCTTAAGTTAGAACTGTATGATGTGACCgc-3′ and 5′-GGTCACATCATACAGTTCTAACTTAAGATATATc-3′ respectively (viral cDNA sequence is in capital letters, a partial *Ksp*I site is underlined, and nonviral sequence is in lowercase letters). The annealed oligonucleotides which formed 5′-terminal *Nco*I and 3′-terminal *Ksp*I cohesive overlaps were ligated to the pCite-L7 cDNA clone which had been digested with both *Nco*I and *Ksp*I (Fig. 2).

Three additional chimeric constructs, 5′Con-pCite-L7, 5′Con-pCite-L20, and 5′Con-pCite-L33, were generated by PCR using Vent DNA polymerase (New England Biolabs) (Fig. 2). These constructs contained nucleotides 1 to 10 of pOSU9 at their 5′ termini and a portion or the complete 3′ NCR of pOSU9 at their 3′ termini. The plus-sense primer used in the PCRs for all three constructs was 5′-taatacgactcactataGGCTTTAAAAattccgg-3′ (the T7 promoter is underlined, viral sequence is in capital letters, and sequence corresponding to nucleotides 11 to 17 in the pCite2a vector is in lowercase letters). The 5′Con-pCite-L7 construct was generated by using the pCite-L7 cDNA clone as a template and a minus-sense primer, 5′-GGTCACAattcggatccga-3′, which is complementary to nucleotides 541 to 560 of the pCite-L7 clone (viral cDNA sequence is in capital letters and nonviral sequence is in lowercase letters). The 5′Con-pCite-L20 and 5′Con-pCite-L33 constructs were generated by using the pCite-L20 and pCite-L33 cDNA clones, respectively, as templates and a minus-sense primer, 5′-GG TCACATCATACAGTTC-3′, which is complementary to the 3′-terminal 18 nt of pOSU9. The PCR products were gel purified, and an aliquot was analyzed on

TABLE 1. Primers used to direct mutation(s) within the 3′-terminal 7 nt of pOSU9-ΔRV

Primer	Sequence[a]	Viral nt
a	5′-CGGAATTAATTTACC-3′	207–221
b	5′-ccgcGGNNNNATCATACAGTTCTA-3′	1043–1062
c	5′-ccgcGGT**ACA**ATCATACAGTTCTA-3′	1043–1062
d	5′-ccgcGGT**GGG**ATCATACAGTTCTA-3′	1043–1062
e	5′-taatacgactcactataGG-3′	1 and 2
f	5′-**AA**TCACATCATACAGTTC-3′	1045–1062
g	5′-G**A**TCACATCATACAGTTC-3′	1045–1062
h	5′-**A**GTCACATCATACAGTTC-3′	1045–1062
i	5′-GG**G**CACATCATACAGTTC-3′	1045–1062
j	5′-GGT**TGTG**TCATACAGTTC-3′	1045–1062

[a] Viral cDNA sequence is in capital letters, and nonviral sequence is in lowercase letters. Mutations are shown in boldface. N indicates either a conservative or a nonconservative mutation. *Ksp*I restriction endonuclease sites and the T7 promoter are underlined.

a 1.2% agarose gel to verify purity. The gel-purified PCR products served as templates for in vitro transcription assays.

Primer-directed mutagenesis of cDNA templates. Templates containing mutations within the 3′-terminal 7 nt were prepared by either of the two methods described below. The first method generated cDNA clones by performing PCR using *Taq* DNA polymerase (Boehringer Mannheim) with pOSU9 as the template and a plus-sense primer corresponding to nt 207 to 221 (primer a [Table 1]) and a minus-sense primer complementary to nt 1043 to 1062 which contained random mutations at positions 1057 to 1060 (primer b [Table 1]). PCR products were gel purified and ligated into the multiple cloning site of the pT7 vector (Novagen). The ligation reactions were used to transform *E. coli* DH5α. Mutations present in the pT7 clones were identified by dideoxynucleotide sequencing. Clones with mutations of interest (derived from primers c and d [Table 1]) were subsequently subcloned by using the following procedure: pT7 clones were digested with *Eco*RV and *Ksp*I, and the insert released from the double digest was gel purified and ligated into pOSU9-ΔRV which had been digested with *Eco*RV and *Ksp*I. The presence of the mutation(s) in the subclones was confirmed by dideoxynucleotide sequencing.

The second method used PCR with Vent DNA polymerase (New England Biolabs) to generate DNA templates for in vitro transcription reactions. Each reaction contained pOSU9-ΔRV as the template with the plus-sense primer corresponding to the T7 promoter (primer e [Table 1]) and the minus-sense primer, complementary to nt 1045 to 1062, containing the desired mutation(s) (primers f to j [Table 1]). The PCR products were gel purified, and an aliquot was analyzed on a 1.2% agarose gel to verify purity.

Preparation of in vitro transcripts. Templates analyzed in the assays were generated by digesting cDNA clones with *Ksp*I followed by removal of the 3′-terminal overhang by using T4 DNA polymerase to create a bona fide rotavirus 3′ terminus. For templates with altered 3′ termini, pOSU9 and pOSU9-Δ*Msc* cDNA clones were digested with either *Bsa*JI, *Bst*UI, and *Hin*dIII to generate OSU9-3′*Bsa*, OSU9-3′*Bst*, OSU9-3′*Hin*, OSU9-Δ*Msc-Bsa*, OSU9-Δ*Msc-Bst*, and OSU9-Δ*Msc-Hin*, respectively (Fig. 1). For templates with 3′-terminal truncations, pOSU9 and pOSU9-ΔRV clones were digested with *Xba*I and *Bfr*I to generate OSU9-3′*Xba*, OSU9-3′*Bfr*, OSU9-ΔRV-3′*Xba*, and OSU9-ΔRV-3′*Bfr*, respectively (Fig. 1). Controls for analyzing the chimeric constructs, Cite-L7-*Eco*RV and Cite-L7-*Sph*, were generated by digesting the pCite-L7 clone with *Eco*RV and *Sph*I, respectively. The 3′ overhang resulting from the *Sph*I digestion was removed by using T4 DNA polymerase. Runoff transcription assays using T7 RNA polymerase were performed as suggested by the manufacturer (Boehringer Mannheim). The assay mixtures were treated with RNase-free DNase for 30 min at 37°C to remove the DNA templates and then extracted with an equal volume of acidic (pH 4.7) phenol-chloroform (5:1). Unincorporated nucleotides were removed by Sephadex G-50 spin chromatography, and the RNA was recovered by ethanol precipitation. The RNA concentration was determined spectrophotometrically. To verify purity, an aliquot was analyzed on a 5% polyacrylamide gel containing 8 M urea, 90 mM Tris, 90 mM boric acid, and 2.5 mM EDTA and detected by silver staining.

In vitro replicase assay conditions. General assay conditions were those described previously (5), with the following modifications: 750 ng of cores, 10 mM magnesium acetate, 1.25 mM each ATP, GTP, and CTP, 0.5 mM UTP, 15 μCi of [α-^{32}P]UTP (specific activity, 3,000 Ci/mmol; New England Nuclear), 2% polyethylene glycol 8000, and a 37°C incubation temperature. The amount of RNA template added per reaction and incubation time varied depending on the type of analysis. Following the desired incubation time, samples were diluted with distilled water, extracted with an equal volume of acidic (pH 4.7) phenol-chloroform (5:1), and precipitated in ethanol. Products and ^{32}P-labeled OSU marker dsRNA were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel) (16) and detected by autoradiography.

(i) Conditions for analyzing templates with internal deletions or altered 3′ termini. For each reaction, 2.77 pmol template (for full-length OSU9 template, 2.77 pmol equals 1 μg) was added to an aliquot taken from an in vitro replicase master mix. After a 4-h incubation, products were analyzed as described for assay type 1 Fig. 1.

Templates were further analyzed to determine if they could replicate in the presence of an equimolar amount of OSU9 template under standard assay conditions (assay type 2 [Fig. 1]). OSU9-ΔRV-3′*Xba* and OSU9-ΔRV-3′*Bfr* templates were used in place of OSU9 for analysis of 3′-terminal truncated templates because of size considerations. For templates with altered 3′ termini, reciprocal reactions were analyzed in parallel. In these experiments, one reaction contained either OSU9-3′*Bsa*, OSU9-3′*Bst*, or OSU9-3′*Hin* template and an equimolar amount of OSU9-Δ*Msc* template and another reaction contained OSU9 template and an equimolar amount of either OSU9-Δ*Msc*-3′*Bsa*, OSU9-Δ*Msc*-3′*Bst*, or OSU9-Δ*Msc*-3′*Hin* template. Duplicate reactions were performed in parallel. After a 4-h incubation, the reactions were analyzed as described above. Gels were scanned on a Betagen Betascope 603 scanner to quantitate the amount of dsRNA synthesized for each template.

(ii) Conditions for analyzing chimeric templates. Ten reactions were analyzed in parallel. In addition to OSU9 template, nine of the reactions contained 2.77 pmol of one of the following templates: OSU9-ΔRV, Cite-L7, 5′Con-Cite-L7, Cite-L7-EcoRV, Cite-L7-Sph, Cite-L20, 5′Con-Cite-L20, Cite-L33, or 5′Con-Cite-L33 template. The 10th reaction contained only the OSU9 template. After a 4-h incubation, products were purified from the reactions and resolved by SDS-PAGE (10% gel). Total counts of the band corresponding to the synthesized dsRNA from each template were obtained by scanning the gel on a Betagen Betascope 603 scanner. The relative replication efficiency (RRE_C) for each template was calculated as total counts in dsRNA produced by chimeric template/total counts in dsRNA produced by Cite-L7 template.

(iii) Conditions for analyzing mutant templates. Aliquots taken from a replicase master mix containing OSU9 template were added to tubes containing 1.38 pmol of mutant, OSU9-ΔRV, or OSU9-ΔRV-3′*Bfr* template. After a 2-h incubation, products were extracted from the reactions and resolved by SDS-PAGE (10% gel). The dsRNA products were quantitated by scanning the gel on a Betagen Betascope 603 scanner. Total counts corresponding to the dsRNA synthesized for a particular template were used directly in the following equation to calculate the relative replication efficiencies (RRE_M): RRE_M = total counts of dsRNA from mutant or control template/total dsRNA counts (OSU9 + template) in sample. The relative replication efficiency for the wild-type OSU9-ΔRV template was normalized to 100%. The factor used to adjust this efficiency for OSU9-ΔRV was used to normalize the relative percentages for the other templates.

RESULTS

Analysis of templates containing internal deletions. Although the NCRs of rotavirus genome segments are presumed to carry the primary *cis*-acting signals for assortment, packaging, and replication, the possibility that these signals are present in the ORF clearly exists. Therefore, a panel of rotavirus strain OSU segment 9 cDNA clones with internal deletions was generated. All of the cDNA clones lack portions of the ORF, with clones pOSU9-ΔRV, pOSU9-Δ*Msc*, exoIII-Δ162, exoIII-Δ108, and exoIII-Δ1025 containing complete 5′ and 3′ NCRs (Fig. 1). Clones pOSU9-Δ3, exoIII-Δ28, and exoIII-Δ37 contain the entire 3′ NCR but lack part of the 5′ NCR, and clone exoIII-Δ1036 contains the entire 5′ NCR but lacks part of the 3′ NCR (Fig. 1). We also made OSU9 transcripts that were truncated or modified at the 3′ terminus following cleavage with the enzymes indicated in Fig. 1. In addition, the transcripts containing identical 3′-terminal truncations or modifications were made from the internally deleted constructs pOSU9-ΔRV and pOSU9-Δ*Msc*. These constructs are not shown in Fig. 1, as they yielded results quantitatively similar to those obtained with truncated or modified OSU9. Synthetic transcripts produced from these clones were tested in the in vitro system under two different assay conditions (assay types 1 and 2). The replication phenotype of each template in each assay was then used to map the *cis*-acting signals required for replication of OSU9. Since the templates used in the analysis differed in size and uridine content, absolute values for the replication efficiencies are not provided in Fig. 1. Instead, we used a scoring procedure which compared, on a molar basis, the amount of dsRNA produced from a template

to the amount of dsRNA produced from the OSU9 template. If the amount of dsRNA produced from a template was greater than 75% of the dsRNA produced from the OSU9 template, it was assigned a + replication phenotype. If the amount of dsRNA produced from a template was between 25 and 75% of the OSU9 dsRNA, it was assigned a < replication phenotype. If the amount of dsRNA produced from a template was less than 25% of the OSU9 dsRNA, it was assigned a − replication phenotype.

For assay type 1, 2.77 pmol of a single template was analyzed under the modified conditions described. All templates containing internal deletions except OSU9-Δ3 replicated as efficiently on a molar basis as the OSU9 template and were assigned a + replication phenotype (Fig. 1). The OSU9-Δ3 template yielded 50% dsRNA compared with the OSU9 template and was assigned a < replication phenotype (Fig. 1). Templates with 3′-terminal truncations, OSU9-3′*Xba* and OSU9-3′*Bfr*, yielded 20% dsRNA compared with the OSU9 template and were assigned a − replication phenotype (Fig. 1).

Assay type 2, a two-template reaction performed with equimolar amounts of OSU9 and template derived from a particular construct, was developed to verify the replication phenotypes determined in assay type 1. All of the internal deletion templates except OSU9-Δ3 replicated to equimolar amounts or higher compared with the OSU9 internal standard and were assigned a + replication phenotype (Fig. 1). The OSU9-Δ3 template replicated to 50% compared with the OSU9 internal standard and was assigned a < replication phenotype (Fig. 1). The templates OSU9-ΔRV-3′*Xba* and OSU9-ΔRV-3′*Bfr* were used in this assay instead of their full-length counterparts because of size considerations. Neither of these templates replicated to detectable levels in the presence of the OSU9 internal standard; therefore, their full-length counterparts, OSU9-3′*Xba* and OSU9-3′*Bfr*, were assigned a − replication phenotype (Fig. 1). Although the 3′-terminally truncated templates replicated to low levels in assay type 1, the dsRNA produced from either template was completely abolished when tested in the presence of an equimolar amount of template containing a complete 3′ NCR. The results from both assay conditions clearly indicated that the 3′-terminally truncated templates lack a *cis*-acting signal critical for replication. Templates with altered 3′ termini were tested in assay type 2 as well. The templates OSU9-Δ*Msc*-3′*Bsa*, OSU9-Δ*Msc*-3′*Bst*, and OSU9-Δ*Msc*-3′*Hin* were tested in the presence of an equimolar amount of OSU9 template. Reciprocal reactions performed with OSU9-3′*Bsa*, OSU9-3′*Bst*, or OSU9-3′*Hin* templates were tested in the presence of an equimolar amount of OSU9-Δ*Msc* template. Figure 1 includes only the replication phenotypes for the OSU9 templates with altered 3′ termini, although quantitatively similar results were obtained for truncated templates with internal deletions as outlined below. The OSU9-Δ*Msc*-3′*Bsa* template, which lacks nt 1062 in OSU9, replicated to 70% compared with the OSU9 internal standard template (Fig. 1 and 3). In a reciprocal reaction, the OSU9-3′*Bsa* template replicated to 50% compared with the OSU9-Δ*Msc* template; therefore OSU9-3′*Bsa* was assigned a < replication phenotype (Fig. 1). The OSU9-Δ*Msc*-3′*Bst* template, which contains an additional guanosine at its 3′ terminus, replicated to 86% compared with the OSU9 internal standard template (Fig. 1 and 3). In a reciprocal reaction, the OSU9-3′*Bst* template replicated to 79% compared with the OSU9-Δ*Msc* internal standard template; therefore, the OSU9-3′*Bst* template was assigned a + replication phenotype. Templates containing five additional nucleotides at the 3′ terminus, OSU9-3′*Hin* and OSU9-Δ*Msc*-3′*Hin*, did not replicate to detectable levels under these conditions (Fig. 1 and 3). The OSU9-3′*Hin* template was assigned a − replication phenotype.

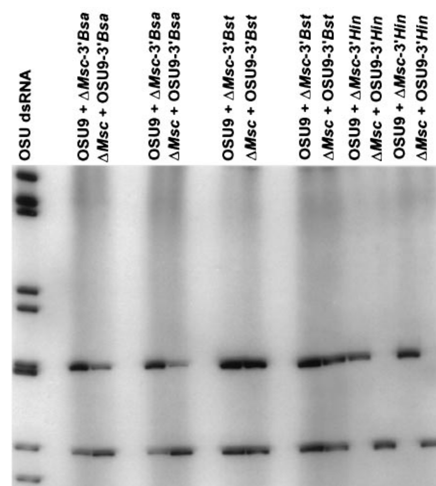

FIG. 3. Autoradiogram of the products of in vitro replication of templates with altered 3′ termini. Two template replication assays are shown. Each reaction mixture contained 2.77 pmol template with native 5′ and 3′ termini and 2.77 pmol template with an altered 3′ terminus, as indicated. Conditions of the assay and analysis are described in Materials and Methods. Shown in the left-most lane are dsRNA markers from rotavirus strain OSU.

Analysis of chimeric constructs. Results from the templates containing the internal deletions and 3′-terminal truncations indicated that essential replication elements lie between nt 1036 and 1062 in the 3′ NCR of OSU9. The phenotypes of the OSU9-Δ3 and exoIII-Δ28 templates suggested that another element, although not absolutely required for RNA replication in vitro, was present between nt 1 and 28 in the 5′ NCR of OSU9. Chimeric cDNA clones in which the complete 3′ NCR of pOSU9, or a portion of it, was ligated to a nonviral sequence were generated. These clones were used as templates in PCRs to generate DNA containing nt 1 to 10 of pOSU9 at their 5′ termini and either all or a portion of the 3′ NCR of pOSU9 at their 3′ termini.

Two chimeric constructs, Cite-L4 and Cite-L7, were made to determine the minimum number of nucleotides required for consistent replication in vitro. The Cite-L4 template contains nt 1059 to 1062 of OSU9 at its 3′ terminus, and the Cite-L7 template contains nt 1056 to 1062 of OSU9 at its 3′ terminus (Fig. 2). Analysis involved three independent experiments in which three reactions, each performed with 2.77 pmol OSU9-ΔRV, Cite-L7, or Cite-L4 template, were analyzed in parallel. When levels of dsRNA synthesized from the chimeric templates were compared with those produced from the OSU9-ΔRV template, values ranged from 14 to 17% for the Cite-L7 template and 0.3 to 4% for the Cite-L4 template (data not shown). These results indicated that the minimum requirement for replication is the 3′-terminal 7 nt that form the rotaviral 3′-terminal consensus sequence.

Additional preliminary experiments involved three reactions each performed with 2.77 pmol Cite-L7, Cite-L7-*Eco*RV, or Cite-L7-*Sph* template. Detectable amounts of dsRNA were reproducibly synthesized from the Cite-L7 template. No product was detected in the parallel reactions performed with either Cite-L7-EcoRV (lacks an L7 insert) or Cite-L7-*Sph* (contains a subterminal L7 insert) template (Fig. 2 and 4). These controls indicated that both the presence and the position of the 3′-terminal consensus sequence play a role in replication.

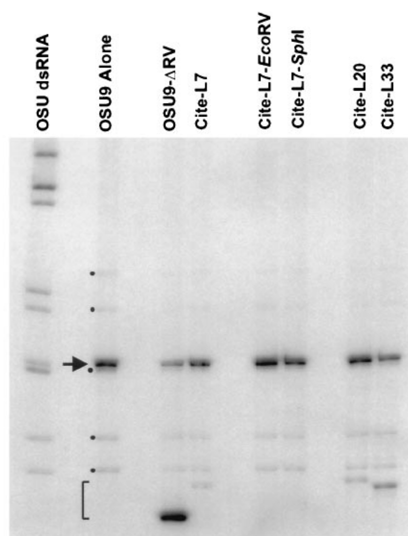

FIG. 4. Autoradiogram of the products of in vitro replication of chimeric templates. Two template reactions are shown. Each reaction mixture contained 40 ng of OSU9 template as an internal control and 2.77 pmol of the indicated chimeric template. Conditions of the replicase assay and analysis of products are described in Materials and Methods. dsRNA from the OSU9 template alone is in the second lane from the left, and dsRNA from rotavirus strain OSU is on the extreme left. The arrow indicates the position of the OSU9 dsRNA control band, and the bracket indicates the positions of the chimeric dsRNA bands. Dots to the left of the OSU9-alone lane indicate the positions of background bands that originate from low-level initiation and synthesis of minus strands on endogenous template that remains within the open cores of some preparations (5). These background bands have slight mobility differences from the OSU dsRNA marker because the open cores were derived from virus strain SA11-4F.

RNA templates derived from chimeric constructs containing increasing amounts of viral 3′ NCR sequence were analyzed to further map the *cis*-acting elements required for rotavirus RNA replication in vitro. All of the chimeric templates consisted of viral sequence placed in the same nonviral backbone, and all were similar in length. The dsRNA produced from each chimeric template was compared with the amount of dsRNA produced from the Cite-L7 template, which contains the minimum signal for replication in vitro. The OSU9 template was present in each reaction to simply serve as an indicator for recovery of dsRNA from the reaction mixture. Previous experiments indicated that the amount of OSU9 template present in each reaction did not interfere with the replication of the chimeric template. Experiments 1A and 1B were performed in parallel, and all samples contained 40 ng of OSU9 template as an internal control. Experiment 2 was a separate analysis in which all samples contained 20 ng of OSU9 template as an internal control. In all three experiments, each reaction contained 2.77 pmol chimeric template in addition to the OSU9 template. The RRE_C calculated for the Cite-L20 template varied, ranging from 1.7 to 4.1. The RRE_C calculated for the Cite-L33 template was more consistent, ranging from 3.6 to 4.9. The inconsistent versus consistent RRE_C values for these two templates could possibly reflect the presence of a complete replication signal in the Cite-L33 template and an incomplete signal in the Cite-L20 template. The RRE_C values for the 5′Con-Cite-L7 and 5′Con-Cite-L20 templates were comparable to those for their Cite-L7 and Cite-L20 counterparts. However, there was over a twofold difference between the ratios of RRE_C obtained for the 5′Con-Cite-L33 template and its Cite-L33 counterpart (Fig. 2 and 4; Table 2).

Analysis of mutant templates. Two lines of evidence suggested that the 3′ terminus plays a critical role in rotavirus

TABLE 2. RRE_C values for chimeric Cite templates

Template	RRE_C[a]		
	Expt 1A	Expt 1B	Expt 2
Cite-L7	1.0	1.0	1.0
5′Con-Cite-L7	ND[b]	1.0	1.0
Cite-L20	1.7	2.1	4.1
5′Con-Cite-L20	ND	1.4	4.9
Cite-L33	3.6	4.7	4.9
5′Con-Cite-L33	ND	9.3	11.7

[a] Calculated as described in Materials and Methods.
[b] ND, not done.

replication. (i) The analysis using the chimeric templates demonstrated that both the presence and the position of the 3′-terminal 7 nt were important for rotavirus replication in vitro (Fig. 4; Table 2). (ii) Adding or deleting one or several nucleotides from the 3′ terminus of either OSU9 or OSU9-Δ*Msc* RNA resulted in detectable decreases in the replication efficiency of the template (Fig. 1 and 3). To further demonstrate the importance of this consensus sequence in rotavirus replication, we analyzed a panel of templates containing mutations in the 3′-terminal region.

The OSU9-ΔRV template yielded readily detectable amounts of dsRNA when analyzed under the two assay conditions used to ascertain the replicase phenotype for the panel of internally deleted templates. This particular template contains both 5′ and 3′ NCRs and lacks only an internal portion of the ORF; thus, the template contains all of the *cis*-acting elements necessary for efficient replication in vitro. Therefore, all mutant DNA constructs were either subcloned into pOSU9-ΔRV or created by PCR using pOSU9-ΔRV as the template. These procedures were performed so that the only difference between these constructs and pOSU9-ΔRV was the mutation(s) present within the 3′-terminal consensus region.

The replication efficiencies of mutant constructs have been calculated from data obtained in four experiments (Table 3). Experiments 1 and 2 were performed independently, whereas experiment 3A and 3B were performed in parallel. Each reaction mixture in experiments 1, 3A, and 3B contained 20 ng of OSU9 template to serve as an internal control. Experiment 2 used 40 ng of OSU9 template per reaction. All experiments included OSU9-ΔRV (3′ terminus, 5′UGUGACC-3′) as a positive control and OSU9-ΔRV-3′*Bfr* as a negative control (Fig. 5). Previous experiments in which these mutant templates were tested in the presence or absence of OSU9 template had been

TABLE 3. RRE_M values for mutant templates

Template	3′-terminal sequence[a]	RRE_M[b] (%)			
		Expt 1	Expt 2	Expt 3A	Expt 3B
1	5′-UGUGACC-3′	100	100	100	100
2	5′-UGUGA**UU**-3′	5	ND[c]	1	8
3	5′-UGUGA**U**C-3′	14	21	20	17
4	5′-UGUGAC**U**-3′	ND	6	15	18
5	5′-UGUG**C**CC-3′	25	26	31	33
6	5′-U**UGU**ACC-3′	ND	33	73	73
7	5′-U**CCC**ACC-3′	17	9	33	32
8	5′-**CACA**ACC-3′	ND	19	16	17
9	OSU9-ΔRV-3′*Bfr*	6	6	3	5

[a] Mutations are shown in boldface.
[b] Calculated as described in Materials and Methods.
[c] ND, not done.

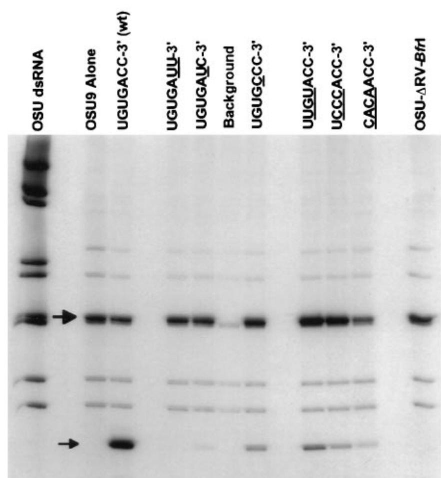

FIG. 5. Autoradiogram of the products of in vitro replication of mutant templates. Two template reactions are shown. Each reaction mixture contained 20 ng of OSU9 template as an internal control and 1.39 pmol of either OSU9-ΔRV, mutant, or OSU9-ΔRV-3'*Bfr* template. Mutations in the terminal sequence are underlined. The background sample contained no exogenous template and indicates the positions of the endogenous dsRNAs sometimes present, as described in the legend to Fig. 4. Assay conditions and analysis are described in Materials and Methods. The position of the dsRNA from the OSU9 alone control is indicated with a large arrow, and marker OSU dsRNA is contained in the left-most lane. The position of dsRNA products of OSU9-ΔRV and mutant templates is indicated with a small arrow.

conducted. The replication values calculated for the mutants tested under either assay condition were virtually the same. The mutant templates were tested in the presence of an internal standard to prevent false positives and to control for variability in replication values due to sample loss. In addition, the use of an internal standard in a quantitative analysis allows direct comparison of replication values obtained from different core preparations which may differ in enzymatic activity. The panel of mutant templates (Table 3) was analyzed to experimentally define the nature of the *cis*-acting signal contained in the 3'-terminal 7 nt. For ease of interpretation, we have designated the templates into three categories. (i) Templates 2, 3, and 4 contain single or double nonconserved mutations at nucleotide positions −1 and −2 from the 3' terminus. These templates analyze the role of the dinucleotide cytidine residues present at the 3' termini of all group A rotavirus genome segments. (ii) Template 5 contains a nonconserved base pair change at position −3 from the 3' terminus. A purine normally resides in this position; for this construct, a cytidine has been substituted for an adenosine. (iii) Templates 6, 7, and 8 analyze the role of positions −4 to −7 from the 3' terminus. These templates contain three or four base pair changes, either all conserved or nonconserved, to determine if these nucleotides must exist as a pattern such as a purine followed by a pyrimidine which is present in OSU9.

The collective results from the category i mutants indicate that the presence of the 3'-terminal cytidine dinucleotide is essential for efficient replication in vitro (Fig. 5; Table 3). The RRE_M for template 2, which contains a dinucleotide uridine sequence, decreased to negative control values. Changing either cytidine in this sequence to a uridine also resulted in a severe reduction in RRE_M (Table 3, templates 3 and 4), although template 3, which has a 3'-terminal cytidine, had slightly higher values than template 4. The RRE_M for the category ii mutant with a nonconserved change at position −3 decreased by approximately 70% compared with the positive control (Fig. 5; Table 3, template 5). The category iii mutants showed the following. The average RRE_M from three experiments analyzing template 6 with conserved changes at nt −4, −5, and −6 was 65% (Fig. 5; Table 3). When this mutant was analyzed with another panel of mutant templates, its RRE_M was approximately 50% (data not shown). Note that this construct contains a pyrimidine followed by a purine pattern, whereas the reciprocal pattern is present in the positive control. The RRE_M was reduced on the average by 77% for template 7, with cytidines at positions −4, −5, and −6 (Table 3; Fig. 5). When this template was analyzed with another panel of mutant templates, its RRE_M was reduced by 85% (data not shown). The RRE_M for template 8, with nonconserved changes at positions −4, −5, −6, and −7, ranged between 16 and 19% (Fig. 5; Table 3). This mutant differs in sequence but retains the purine followed by a pyrimidine pattern which is present in the positive control. There was more flexibility for the template with conserved changes at positions −4, −5, and −6 than for templates with nonconserved changes even when these changes retained a purine followed by a pyrimidine pattern. This result indicated that the requirement for nt −4, −5, and −6 is not merely a pattern of a purine followed by pyrimidine but may be related to primary sequence.

DISCUSSION

We used the in vitro replicase system to identify the *cis*-acting signals essential for in vitro replication of OSU9 mRNA. We have semiquantitatively determined the replicase phenotypes of a panel of internally deleted or 3'-terminally truncated templates by analyzing their replication under two different assay conditions (Fig. 1). The 3'-truncated templates, OSU9-3'*Xba* and OSU9-3'*Bfr*, replicated to low levels in single-template assays using 2.77 pmol template (Fig. 1, assay type 1); however, the dsRNA produced from either template was abolished when tested in the presence of an equimolar amount of template containing a complete 3' NCR, suggesting that their replication was nonspecific (Fig. 1, assay type 2). All templates containing internal deletions except the OSU9-Δ3 template were assigned + replication phenotypes in assay types 1 and 2 (Fig. 1). The OSU9-Δ3 template, which contains nonviral sequence at nt 3 to 11, was assigned a < replication phenotype in assay types 1 and 2 (Fig. 1). The phenotypes of the OSU9-Δ3 and exoIII-Δ28 templates suggested that a signal which contributes to replication was located between nt 1 and 28. We conclude that the 3' terminus of the mRNA contains a critical *cis*-acting signal for replication whereas the 5' terminus may contain a replication-enhancing signal. The internal deletions allowed us to map the regions essential for efficient RNA replication in vitro to nt 1 to 28 and 1036 to 1062 of the OSU9 reporter template.

Experiments with chimeric templates revealed that the 3'-terminal 7 nt (nt 1056 to 1062) of the OSU9 template formed the minimum *cis*-acting signal for replication in vitro, a signal that we call the minimal promoter (Fig. 4; Table 2). Control constructs demonstrated that the minimal promoter must be present at the 3' terminus for a template to be replication competent. Chimeric templates that contained increasing amounts of the OSU9 3' NCR (Cite-L20 and Cite-L33, respectively) were better templates than Cite-L7 (Fig. 4; Table 2), suggesting that a second signal may exist within the 3' NCR upstream of the minimal promoter. If the increases in relative replication efficiency with larger portions of the 3' NCR are due to specific interaction between the template and the replicase, two distinct signals may exist within the 3' NCR of an mRNA template: (i) the 3'-terminal 7 nt constituting the min-

imal promoter of synthesis and (ii) a second enhancing signal upstream between nt 1029 and 1056. However, one must note that the relative replication efficiencies for the chimeric templates were proportional to the amount of viral sequence present in the template. Since the in vitro system was optimized with full-length OSU9 template (5), the relative increases may result from simple restoration of viral sequences for which the system is optimized. We do, however, favor the notion that the 3′-terminal signal is bipartite. Addition of the 5′-terminal consensus sequence to chimeric template consistently increased the replication efficiency of templates which contained the entire 3′ NCR (5′Con-Cite-L33) but had little effect if only the last 7 or 20 nt of the 3′ NCR were present (5′Con-Cite-L7 and 5′Con-Cite-L20; Table 2). The role of the 5′-terminal consensus sequence is not clear, but it may help stabilize an RNA-protein interaction or enable the RNA to form a structure conducive to more efficient replication, or both termini may interact with the replicase. Experiments testing the biological significance of the 5′-terminal consensus sequence in RNA replication are in progress.

Examination of specific mutations within the 3′-terminal 7 nt of the minimal promoter revealed the following. The cytidine residues at positions −1 and −2 were essential for replication; nonconservative changes of these residues to uridine abolished replication (Table 3, template 2). A much smaller effect on replication efficiency was observed when positions −1 and −2 were nonconservatively changed to uridine separately (Table 3, templates 3 and 4). We conclude that the presence of a 3′-terminal CC dinucleotide is essential for efficient replication, a conclusion supported by absolute conservation of C at these positions among all group A rotavirus mRNAs. Position −3 can be either A or G in rotavirus RNAs and is an A in OSU9. The nonconservative change of position −3 to the pyrimidine C resulted in a 75% reduction in replication efficiency (Table 3, template 5), suggesting that a purine in this position is required for optimal activity. There was more flexibility for the template with conservative changes at nt −4 to −6 (Table 3, template 6) than for a template with nonconservative changes at nt −4 to −7 (Table 3, template 8) even when these changes retained a purine followed by a pyrimidine pattern. Although this alternating purine-pyrimidine pattern is present at nt −4 to −7 of OSU9, it must not constitute a signal for replication. Guanosines and uridines are conserved in this region, indicating that either nucleotide or both nucleotides may be important for replication, although their positions relative to the highly conserved residues (e.g., −1, −2, and −7) can be somewhat relaxed. These nucleotides must influence the nature of the replication signal because the template containing cytidines at nt −4 to −6 (Table 3, template 7) contained the highly conserved nucleotides but suffered a severe reduction in replication efficiency.

Our data, taken together, suggest that the *cis*-acting signal for replication of the OSU9 template is tripartite. (i) The most critical and essential portion of the *cis*-acting signal is the minimal promoter of minus-strand synthesis in the 3′-terminal 7 nt of OSU9. Interestingly, this sequence is conserved on all group A rotavirus mRNAs, suggesting that these sequences constitute a universal replication signal for all group A genome segments. (ii) A second signal, which lies immediately upstream of the minimal promoter, between nt 1029 and 1056, enhances synthesis initiated at the minimal promoter. The fact that this sequence lies within the 3′ NCR, which differs in size and sequence among the different genome segments but is well conserved for a particular genome segment, suggests a segment-specific replication signal. Whether such segment-specific regulation of replication occurs is unknown, since equimolar amounts of rotavirus dsRNA are found in subviral complexes isolated from infected cells (11, 22). (iii) A third signal, which enhances replication, is located at the 5′ terminus of the OSU9 template. The 5′-terminal 10 nt of the template will confer enhancing activity on replication mediated by the other two signals. However, we do not yet know if the entire 5′-terminal signal resides in nt 1 to 10, as we have not yet examined chimeric constructs with larger portions of the 5′ NCR and we have not yet deleted closer than 27 nt to the 5′ terminus. However, it is again interesting to note that nt 1 to 10 constitute the 5′-terminal conserved sequence present on all group A rotavirus mRNAs. The observation of similar signal organizations and locations for two rotavirus templates derived from different genome segments of different virus strains (this work and reference 23) suggests that this organization of replication signals may be universal for all rotavirus templates.

Similar studies to identify *cis*-acting signals regulating replication of rotavirus RNA have been carried out using SA11 genome segment 8 (SA11-8) templates (23). These studies also identified a tripartite *cis*-acting replication signal. The most important of the signals was the absolutely required minimal promoter, which was localized to the 3′-terminal 7 nt of the SA11-8 template. A second signal was located near the 5′ end of the 3′ NCR, and the third signal was localized in the 5′ NCR. The tripartite nature of the *cis*-acting replication signal and locations of the signal are very similar to those that we found in OSU9.

Similar multipartite *cis*-acting replication signals have been identified in the dsRNA bacteriophage ϕ6 and in the dsRNA virus of yeast L-A. In ϕ6, the template RNA must be packaged before it is replicated; thus, packaging signals are a prerequisite for replication (9, 12). Packaging sites have been mapped to the 5′ NCRs (13), and replication signals have been mapped to the 3′ termini of the three ϕ6 genome segments (12). In the yeast L-A virus, the multipartite *cis*-acting signals required for in vitro replication include (i) the 3′-terminal 4 nt, (ii) a stem-loop structure adjacent to the 3′ terminus, and (iii) another stem-loop structure some 400 nt from the 3′ terminus of the template. The stem-loop distal from the 3′ terminus overlaps with the packaging site used in vitro (10, 32).

Similar multipartite *cis*-acting replication signals have been identified in single-stranded RNA viruses. For example, in a vesicular stomatitis virus replication system, the 3′-terminal 51 nt, together with the 5′-terminal 51 nt, of the vesicular stomatitis virus genome conferred the ability to be replicated, assembled into nucleocapsids, and encapsidated on a template RNA (21). In influenza virus, the 3′-terminal 11 nt fulfilled the minimum requirement for virion RNA synthesis in vitro (17). Studies with influenza virus have also indicated that the structure of the RNA template may help to regulate enzymatic activity during replication (7, 18), and recent in vitro studies have indicated that the viral polymerase may interact specifically with both 5′ and 3′ termini in the initiation of transcription (8, 14).

Finally, it is of interest that the minimal promoter of rotavirus minus-strand synthesis (this work and reference 23) overlaps with the binding site identified for the rotavirus nonstructural single-stranded RNA-binding protein NSP3 (25, 26). NSP3 was found to bind to the 3′-terminal 4 or 5 nt, depending on the assay used, and mutagenesis studies showed that the binding of NSP3 was sequence specific. In the infected cell, NSP3 is distributed in a filamentous pattern (19) suggesting association with the cytoskeleton. This finding suggests that NSP3 may be associated with replication of rotavirus RNA templates, by mediating the transport of templates to viroplasms where replication is believed to occur (24). Thus, the 3′

consensus sequence may serve as an element both for selection of cytoplasmic viral mRNA for transport to the viroplasm and for replication once the mRNA encounters the replicase in the viroplasm.

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